IDENTIFICATION AND CHARACTERIZATION OF THE PYRIMIDINE BIOSYNTHETIC OPERON IN Streptomyces griseus

THESIS

Presented to the Graduate Council of the University of North Texas in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

By

Jody J. Hooten, B.A.
Denton, Texas
May, 1998
Hooten, Jody J., Identification and characterization of the pyrimidine biosynthetic operon in *Streptomyces griseus*. Master of Science (Molecular Biology), May, 1998, 78 pp., 2 tables, 12 illustrations, references, 52 titles.

To further understand the ATCase/DHOase bifunctional complex formed in *Streptomyces*, the genes encoding these and other pyrimidine enzymes were identified and characterized. Polymerase chain reaction (PCR) was utilized in this effort. Primers were constructed by selecting conserved regions of pyrimidine genes from known gene and protein sequences of a wide variety of organisms. These sequences were then optimized to *Streptomyces* codon usage. PCR products were obtained from internal sites within pyrimidine genes and also from primer combinations of different genes. The size, orientation, and partial sequence of the resulting products shows that *Streptomyces* has a gene organization of pyrR followed by pyrB, pyrC, carA, carB, and pyrF in an operon similar to that found in other Gram-positive bacteria.
IDENTIFICATION AND CHARACTERIZATION OF THE PYRIMIDINE
Biosynthetic Operon in Streptomyces griseus

THESIS

Presented to the Graduate Council of the University of North Texas in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

By

Jody J. Hooten, B.A.

Denton, Texas

May, 1998
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>v</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Pyrimidine biosynthetic pathway</td>
<td>4</td>
</tr>
<tr>
<td>Genetic organization</td>
<td>5</td>
</tr>
<tr>
<td>Streptomyces</td>
<td>10</td>
</tr>
<tr>
<td>METHODS</td>
<td>15</td>
</tr>
<tr>
<td>Bacterial strains</td>
<td>15</td>
</tr>
<tr>
<td>Media and growth conditions</td>
<td>15</td>
</tr>
<tr>
<td><em>Streptomyces</em> spore suspension</td>
<td>16</td>
</tr>
<tr>
<td>Harvesting of bacterial cultures</td>
<td>17</td>
</tr>
<tr>
<td>Preparation of competent cells for transformation</td>
<td>18</td>
</tr>
<tr>
<td>Agarose minigel electrophoresis</td>
<td>19</td>
</tr>
<tr>
<td>Isolation of chromosomal DNA</td>
<td>20</td>
</tr>
<tr>
<td>Design of oligonucleotides for polymerase chain reaction</td>
<td>22</td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td>24</td>
</tr>
<tr>
<td>Purification of PCR products</td>
<td>29</td>
</tr>
<tr>
<td>Cloning of purified PCR products</td>
<td>32</td>
</tr>
<tr>
<td>Isolation of plasmid DNA by alkaline lysis</td>
<td>34</td>
</tr>
<tr>
<td>Analysis of plasmid DNA by restriction enzyme digestion</td>
<td>37</td>
</tr>
<tr>
<td>Preparation of plasmid DNA for sequencing</td>
<td>38</td>
</tr>
<tr>
<td>Double stranded DNA sequencing</td>
<td>40</td>
</tr>
<tr>
<td>Preparation of polyacrylamide sequencing gels</td>
<td>44</td>
</tr>
<tr>
<td>Loading and electrophoresing the sequencing gel</td>
<td>46</td>
</tr>
<tr>
<td>Autoradiography of sequencing gels</td>
<td>48</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td>49</td>
</tr>
<tr>
<td>Polymerase chain reaction products</td>
<td>49</td>
</tr>
<tr>
<td>Partial sequence analysis of the pyrimidine operon</td>
<td>59</td>
</tr>
<tr>
<td>in <em>S. griseus</em></td>
<td></td>
</tr>
<tr>
<td>Genetic organization</td>
<td>67</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>70</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Linkage map locations of pyrimidine genes in E. coli, S. typhimurium, and P. aeruginosa.</td>
<td>6</td>
</tr>
<tr>
<td>2. Oligonucleotide primers for PCR.</td>
<td>25</td>
</tr>
</tbody>
</table>
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pyrimidine biosynthetic pathway.</td>
<td>2</td>
</tr>
<tr>
<td>2. Schematic diagram of low G+C content, Gram-positive bacterial pyrimidine operons</td>
<td>8</td>
</tr>
<tr>
<td>3. Schematic diagram of high G+C content bacterial pyrimidine operons</td>
<td>11</td>
</tr>
<tr>
<td>4. Internal pyrimidine gene products from PCR of <em>S. griseus</em> 10137 DNA</td>
<td>50</td>
</tr>
<tr>
<td>5. <em>S. griseus</em> 10137 PCR products which span two or more pyrimidine genes and originate within <em>pyrR</em></td>
<td>52</td>
</tr>
<tr>
<td>6. <em>S. griseus</em> 10137 PCR products which span two or more pyrimidine genes</td>
<td>54</td>
</tr>
<tr>
<td>7. <em>M. tuberculosis</em> pyrimidine operon with PCR primer locations and orientations</td>
<td>56</td>
</tr>
<tr>
<td>8. Probable <em>Streptomyces</em> pyrimidine operon with PCR primer locations and orientations</td>
<td>60</td>
</tr>
<tr>
<td>9. Partial nucleotide and corresponding AA sequence of the 282 bp <em>pyrB</em> PCR product</td>
<td>63</td>
</tr>
<tr>
<td>10. Amino acid comparison of 20 AA’s from the <em>S. griseus</em> ATCase with several other known bacterial ATCases</td>
<td>64</td>
</tr>
<tr>
<td>11. Partial nucleotide and corresponding AA sequence of the <em>pyrR</em> gene of <em>S. griseus</em></td>
<td>65</td>
</tr>
<tr>
<td>12. Amino acid comparison of 20 AA’s from the UPRTase of <em>S. griseus</em> with several other known UPRTases</td>
<td>66</td>
</tr>
</tbody>
</table>
INTRODUCTION

The study of pyrimidine biosynthesis is important due to the role that pyrimidines play in forming the genetic material that is necessary for cellular growth and for the passing of genetic information to subsequent generations. Pyrimidines serve as building blocks in the informational macromolecules ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). Pyrimidines are found in all organisms and are six-membered, aromatic heterocyclic ring compounds. A pyrimidine nucleoside consists of nitrogenous bases, cytosine and thymine in DNA and cytosine and uracil in RNA, linked to a pentose sugar. In DNA the pentose is 2'-deoxyribose, whereas in RNA it is ribose. Nucleotides are nucleosides with a phosphate group added.

The biosynthesis of uridine-5'-monophosphate (UMP; Fig. 1), which itself serves as a precursor for all the pyrimidine nucleotides, is accomplished in six enzymatic steps referred to as the pyrimidine biosynthetic pathway. This pathway appears to be universal and follows the same sequence in all organisms thus far studied including bacteria, fungi, plants, and animals (O’Donovan & Neuhard, 1970; Grogan & Gunsalus, 1993).
Figure 1. Pyrimidine biosynthetic pathway in *Escherichia coli* and *Salmonella typhimurium*. Gene symbols and the enzymes they encode are: *ndk* - nucleoside diphosphate kinase; *pyrA* (*carAB*) - carbamoylphosphate synthetase; *pyrBI* - aspartate transcarbamoylase; *pyrC* - dihydroorotase; *pyrD* - dihydroorotate dehydrogenase; *pyrE* - orotate phosphoribosyltransferase; *pyrF* - OMP decarboxylase; *pyrG* - CTP synthetase; *pyrH* - UMP kinase. Adapted from Neuhard & Nygaard (1987).
Pyrimidine biosynthetic pathway.

The enzyme carbamoylphosphate synthetase (CPSase, EC 6.3.5.5) catalyzes the first step in pyrimidine synthesis. The reaction utilizes bicarbonate, ammonium ions or glutamine, and two molecules of adenosine-5'-triphosphate (ATP) in the formation of one molecule of carbamoylphosphate and adenosine-5'-diphosphate (ADP) (Anderson & Meister, 1965; Kalman et al., 1966). Carbamoylphosphate is required for both arginine and pyrimidine synthesis (Abdelal et al., 1969).

The first committed step in pyrimidine biosynthesis is the production of carbamoylaspartate (CAA) by aspartate transcarbamoylase (ATCase, EC 2.1.3.2). Aspartate is carbamoylated at the amino group, producing CAA and releasing inorganic phosphate. The enzyme dihydroorotase (DHOase, EC 3.5.2.3) then catalyzes the cyclization of CAA, resulting in the release of a molecule of water and production of dihydroorotate (DHO).

Following this, DHO is oxidized to orotate (OA) in a reaction catalyzed by dihydroorotate dehydrogenase (DHOdehase, EC 1.3.3.1). The first pyrimidine nucleotide is then produced by the transfer of ribose-5'-phosphate from 5'-phosphoribosyl-1'-pyrophosphate (PRPP) to OA to form orotidine-5'-monophosphate (OMP), a reaction catalyzed by orotate phosphoribosyltransferase (OPRTase, EC 2.2.4.10). OMP is decarboxylated by the enzyme OMP decarboxylase
(OMPdecase, EC 4.1.1.23) in the final step in the production of UMP.

UMP serves as a precursor for the production of the pyrimidine nucleoside triphosphates, uridine-5'-triphosphate (UTP) and cytidine-5'-triphosphate (CTP). In sequential steps, UMP is first phosphorylated to uridine-5'-diphosphate (UDP) by the highly specific UMP kinase (EC 2.7.4.4). A non-specific enzyme, nucleoside diphosphate kinase (NDK, EC 2.7.4.6), further phosphorylates UDP to form UTP. CTP synthetase (EC 6.3.4.2) is responsible for conversion of UTP to CTP by transferring an amino group from glutamine.

Genetic organization.

Although the sequence of enzymatic reactions in the pyrimidine pathway is virtually the same, organization of the pyrimidine genes varies in different organisms. In many bacterial systems, the six enzymatic steps of pyrimidine biosynthesis are encoded by unlinked genes. In the Gram-negative bacteria of low guanine + cytosine (G+C) content, such as *Escherichia coli* K-12 and *Salmonella typhimurium*, the pyrimidine genes are scattered throughout the genome (Table 1).

Likewise, the genes in the Gram-negative, high G+C content genus *Pseudomonas* are found at different chromosomal locations (Holloway et al., 1990; Holloway et al., 1994) (Table 1). However, in both *P. putida* and *P. aeruginosa*,
Table 1. Linkage map locations of pyrimidine genes in *E. coli*, *S. typhimurium*, and *P. aeruginosa* (Bachmann, 1987; Sanderson & Hurley, 1987; Neuhard & Kelln, 1996; Holloway et al., 1994; D. Brichta, personal communication).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Map location (minutes)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td><em>S. typhimurium</em></td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td>carAB/pyrA</td>
<td>0.6, 0.7</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>pyrBI (pyrBC')</td>
<td>96.3</td>
<td>98</td>
<td>19.5</td>
</tr>
<tr>
<td>pyrC</td>
<td>24.2</td>
<td>23</td>
<td>34.5</td>
</tr>
<tr>
<td>pyrD</td>
<td>21.6</td>
<td>20</td>
<td>41.5</td>
</tr>
<tr>
<td>pyrE</td>
<td>82.1</td>
<td>79</td>
<td>11.0</td>
</tr>
<tr>
<td>pyrF</td>
<td>28.8</td>
<td>33</td>
<td>41.5</td>
</tr>
</tbody>
</table>

The scaffolding subunit of ATCase is a catalytically-inactive, DHOase-like protein which is encoded by a gene immediately downstream of the ATCase catalytic subunit gene, *pyrB* (Fig. 3). In fact, there is a four base pair overlap of these two open reading frames (Schurr, 1992; Vickrey, 1993). The catalytically active DHOase is encoded by a separate *pyrC* located next to *argG* on the chromosome of *P. aeruginosa* (D. Brichta, personal communication).

In *Bacillus*, a Gram-positive bacterium with low G+C content, the genes for pyrimidine biosynthesis are organized into a single operon (Quinn et al., 1991). The segment of the *B. subtilis* chromosome containing this gene cluster has been sequenced and found to contain eight overlapping cistrons encoding the six enzymes of pyrimidine biosynthesis, as well as genes for uracil permease (*pyrP*)
and a regulatory protein (pyrR) (Turner et al., 1994; Kahler & Switzer, 1996) (Fig. 2). A similar genetic arrangement has been found in B. caldolyticus (Ghim et al., 1994; Ghim & Neuhard, 1994) and in Enterococcus faecalis (Li et al., 1995).

Lactobacillus plantarum, a Gram-positive bacterium with low G+C content, displays a genetic organization similar to Bacillus. However, the pyrimidine operon in L. plantarum lacks pyrP and pyrDb (Elagoz et al., 1996). The operon in L. leichmannii, on the other hand, includes only pyrB, pyrC, and a gene with unknown function referred to as gene x in its operon (Schenk-Groninger et al., 1995). Clostridium acetobutylicum, another Gram-positive bacterium with low G+C content, contains an operon similar to that of other Gram-positive bacteria in that pyrDb, pyrDa, and pyrF are situated adjacent to each other (Genome Therapeutics Corp., available on the Internet at http://pandora.cric.com/htdocs/sequences/clostridium/clospage.html). However, C. acetobutylicum appears to be unique in that it has a pyrB and pyrI upstream of these three genes within the operon.

Other Gram-positive, low G+C content bacteria in which pyrimidine operons have been found include Lactococcus lactis (Andersen et al., 1996) and Streptococcus pneumoniae (TIGR Microbial Database, available on the Internet at http://www.tigr.org/tdb/mbd/mbd.html).
Figure 2. Schematic diagram of low G+C content, Gram-positive bacterial pyrimidine operons (not drawn to scale). Overlapping genes are shown on separate lines. The *Enterococcus faecalis* and *Streptococcus pneumoniae* gene overlaps are hypothetical and currently unknown. Gene symbols and the enzymes they encode are: \( x \) - unknown; \( \text{pyrAA} \) (equivalent to \( \text{carA} \)) - glutaminase; \( \text{pyrAB} \) (equivalent to \( \text{carB} \)) - carbamoylphosphate synthetase; \( \text{pyrB} \) - aspartate transcarbamoylase; \( \text{pyrC} \) - dihydroorotase; \( \text{pyrD}, \text{pyrDa} \) (equivalent to \( \text{pyrDI} \)), \( \text{pyrDb} \) (equivalent to \( \text{pyrDII} \)) - dihydroorotate dehydrogenase; \( \text{pyrE} \) - orotate phosphoribosyltransferase; \( \text{pyrF} \) - OMP decarboxylase; \( \text{pyrI} \) - ATCase regulatory subunit; \( \text{pyrK} \) - involved with electron transport; \( \text{pyrP} \) - uracil permease; \( \text{pyrR} \) - regulatory protein. Adapted from: *Bacillus subtilis* (Turner et al., 1994; Kahler and Switzer, 1996); *B. caldolyticus* (Ghim et al., 1994); *E. faecalis* (Li et al., 1995); *Lactobacillus plantarum* (Elagoz et al., 1996); *L. leichmannii* (Schenk-Groninger et al., 1995); *Clostridium acetobutylicum* (Genome Therapeutics Corp., available on the Internet at http://pandora.cric.com/htdocs/sequences/clostridium/clospage.html); *Lactococcus lactis* (Andersen et al., 1996); and *S. pneumoniae* (TIGR Microbial Database, available on the Internet at http://www.tigr.org/tdb/mdb/mdb.html).
Pyrimidine Operons of Low G+C, Gram+ Bacteria

*Bacillus subtilis* and *B. caldolyticus*

- *pyrR*  
- *pyrP*  
- *pyrB*  
- *pyrC*  
- *pyrAA*  
- *pyrAB*  
- *pyrDb*  
- *pyrDa*  
- *pyrF*  
- *pyrE*  

*Enterococcus faecalis*

- *pyrR*  
- *pyrP*  
- *pyrB*  
- *pyrC*  
- *pyrAA*  
- *pyrAB*  
- *pyrDb*  
- *pyrDa*  
- *pyrF*  
- *pyrE*  

*Lactobacillus plantarum*

- *pyrR*  
- *pyrB*  
- *pyrC*  
- *pyrAA*  
- *pyrAB*  
- *pyrD*  
- *pyrF*  
- *pyrE*  

*Lactobacillusleichmannii*

- *pyrB*  
- *pyrC*  
- *x*  

*Clostridium acetobutylicum*

- *pyrB*  
- *pyrI*  
- *pyrDb*  
- *pyrDa*  
- *pyrF*  

*Lactococcus lactis*

- *pyrK*  
- *pyrDb*  
- *pyrF*  

*Streptococcus pneumoniae*

- *pyrR*  
- *pyrB*  
- *pyrAA*  
- *pyrAB*  

- *pyrK*  
- *pyrDb*  
- *pyrF*  
- *pyrE*
A Gram-negative extreme thermophile with a high G+C content, *Thermus aquaticus*, has an operon similar to that of *Pseudomonas aeruginosa* and *P. putida* with a few exceptions. Unlike these two pseudomonads, *T. aquaticus* has a *pyrC* that encodes an active DHOase within its pyrimidine operon in addition to a unique open reading frame between *pyrB* and *pyrC* designated *bbc* that encodes a 17 kDa protein (Van de Casteele et al., 1997).

To date there is very little information on pyrimidine operons or indeed *pyr* genes in the high G+C content, Gram-positive bacteria. However, sequence data on *Mycobacterium tuberculosis* and *M. leprae* have shown the presence of an operon similar to that of *Bacillus* but lacking the *pyrP*, *pyrDa*, *pyrDb*, and *pyrE* genes that are found in the *Bacillus* operon (Phillipp et al., 1996). In addition, *Mycobacterium* has an open reading frame, designated *orf1*, between *pyrC* and *pyrAA* (Fig. 3).

**Streptomyces.**

Pyrimidine metabolism has not been studied extensively in one important order, the Actinomycetes. This order contains a wide range of genera and species which are generally divided into eight broad families based on partial sequencing of 16S rRNA. Of these families, only one, the family *Streptomycetaceae*, does not contain diverse taxa
Figure 3. Schematic diagram of high G+C content bacterial pyrimidine operons (not drawn to scale). Overlapping genes are shown on separate lines. Gene symbols and the enzymes they encode are: \texttt{bbc, orfl} - unknown; \texttt{pyrAA} (equivalent to \texttt{carA}) - glutaminase; \texttt{pyrAB} (equivalent to \texttt{carB}) - carbamoylphosphate synthetase; \texttt{pyrB} - aspartate transcarbamoylase; \texttt{pyrC} - dihydroorotase; \texttt{pyrC'} - inactive dihydroorotase-like; \texttt{pyrF} - OMP decarboxylase; \texttt{pyrR} - regulatory protein. Adapted from: \textit{Mycobacterium leprae} and \textit{M. tuberculosis} (Phillipp et al., 1996); \textit{Pseudomonas aeruginosa} (Vickrey, 1993); \textit{P. putida} (Shurr et al., 1995); and \textit{Thermus aquaticus} (Van de Casteele et al., 1997).

\textit{Mycobacterium tuberculosis} and \textit{M. leprae}

\begin{center}
\begin{tabular}{cccccccc}
\texttt{pyrR} & \texttt{pyrB} & \texttt{pyrC} & \texttt{orfl} & \texttt{pyrAA} & \texttt{pyrAB} & \texttt{pyrF} \\
\end{tabular}
\end{center}

\textit{Pseudomonas aeruginosa} and \textit{P. putida}

\begin{center}
\begin{tabular}{ccc}
\texttt{pyrR} & \texttt{pyrB} & \texttt{pyrC'} \\
\end{tabular}
\end{center}

\textit{Thermus aquaticus}

\begin{center}
\begin{tabular}{cccc}
\texttt{pyrR} & \texttt{pyrB} & \texttt{bbc} & \texttt{pyrC} \\
\end{tabular}
\end{center}
(Goodfellow, 1989). This family contains a number of genera, the most widely known being Streptomyces.

Streptomyces exhibits a complex life cycle. Septation and fragmentation of hyphae results in development of spores which can be found in or on the mycelium. Germination of spores results in formation of vegetative mycelia characterized as a branching network of multinucleoid hyphae, occasionally interrupted by septa (Chater, 1993). As the supply of nutrients decreases, vegetative mycelia begin to produce aerial mycelia. Septation of aerial mycelia results in formation of chains of uninucleoid spores.

The genus Streptomyces was proposed in 1943 by Waksman & Henrici for aerobic, spore-forming actinomycetes which had been previously termed Actinomyces. The streptomycetes are filamentous soil bacteria found within the high G+C Gram-positive subdivision based on 16S rRNA homology (Woese, 1987). They are most notable for their large genome size of approximately $8 \times 10^6$ bp (Lezhava et al., 1995; Kieser et al., 1992; Leblond et al., 1993; Leblond et al., 1990), their DNA base composition of 69 to 78 mol % guanine (G) plus cytosine (C) (Korn-Wendisch & Kutzner, 1992), and the large number of secondary metabolites they produce including vitamins, hydrolytic enzymes, herbicides, enzyme inhibitors, and over 70% of naturally occurring antibiotics (Berdy, 1980; Goodfellow & Cross, 1983). The industrial and academic importance of these products has generated
widespread interest in the isolation and study of the streptomycetes. In addition, Streptomyces also stands out as one of only a few prokaryotes in which a linear chromosome is present (Hinnebusch & Tilly, 1993; Lin et al., 1993; Lezhava et al., 1995). This linear chromosome is found as a single copy in spores. However, multiple copies of the chromosome are present in vegetative and aerial mycelia (Leblond et al., 1993).

The application of polymerase chain reaction (PCR) to obtain rDNA sequences has dramatically extended the potential for use of these sequences to elucidate natural relationships between organisms (Stackebrandt et al., 1992). In the genus Streptomyces, Stackebrandt and colleagues observed that the complete 16S rRNA sequences of many species were so similar that analysis was not practical. Instead, analysis was restricted to two regions of the sequence, one for species-specific signatures and another for intragenic classification (Stackebrandt et al., 1992; Kim et al., 1993). Due to the limitations of highly similar 16S rRNA sequences, analysis of these sequences cannot be used to solve all problems of streptomycete taxonomy. Instead, analysis of rRNA must be used in combination with other sources of information in the determination of streptomycete evolution (Stackebrandt et al., 1992). To that end, analysis of pyrimidine gene organization, in
conjunction with 16S rRNA data, should provide a more complete picture of bacterial evolution.

A representative species, *Streptomyces griseus*, was chosen for study. *S. griseus* was one of the original antibiotic-producing organisms discovered by Waksman.

In order to better understand pyrimidine metabolism within the genus *Streptomyces* and its possible evolutionary significance, study of pyrimidine gene organization was undertaken.
METHODS

Bacterial strains.

The wild-type strain *Streptomyces griseus* (ATCC 10137) was obtained from the American Type Culture Collection (ATCC). *Escherichia coli* DH5α was obtained from Life Technologies (Grand Island, NY).

Media and growth conditions.

*Streptomyces* spore suspensions were obtained from growth on sporulation agar plates (Hopwood et al., 1985). Sporulation agar contained 1 g l\(^{-1}\) yeast extract, 1 g l\(^{-1}\) beef extract, 2 g l\(^{-1}\) tryptose, trace FeSO\(_4\), 10 g l\(^{-1}\) glucose, and 15 g l\(^{-1}\) agar in distilled, deionized water (ddH\(_2\)O). All *Streptomyces* cultures were streaked for purity on Difco nutrient agar containing 3 g l\(^{-1}\) beef extract, 5 g l\(^{-1}\) peptone, and 15 g l\(^{-1}\) agar in H\(_2\)O. The *E. coli* DH5α culture was grown in Luria-Bertani (LB) broth (10 g l\(^{-1}\) Difco Bacto-Tryptone, 5 g l\(^{-1}\) Difco Yeast Extract, and 10 g l\(^{-1}\) sodium chloride) or on LB agar (15 g l\(^{-1}\) Difco Bacto Agar).

For isolation of total DNA, 10-50 μl of an *S. griseus* spore suspension was inoculated into 100 ml of *Streptomyces* minimal liquid medium (Hopwood et al., 1985) as a starter culture. This minimal liquid medium contained 2 g l\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 5 g l\(^{-1}\) Difco Casamino acids, 0.6 g l\(^{-1}\) MgSO\(_4\).7H\(_2\)O,
and 50 g l⁻¹ polyethyleneglycol (PEG) 8000 in 800 ml ddH₂O. To this, 1 ml of minor elements solution (per liter: 1 g ZnSO₄·7H₂O, 1 g FeSO₄·7H₂O, 1 g MnCl₂·4H₂O, and 1 g CaCl₂, anhydrous) was added. After autoclaving, 150 ml final volume of 0.1 M NaH₂PO₄/K₂HPO₄ buffer, pH 6.8, and 20 mM succinate, pH 7.0, as carbon source was included. The starter culture was incubated with vigorous shaking (200 rpm) at 30 °C for 3-4 days. Approximately 1 ml of the starter culture was then inoculated into 500 ml of nutrient broth with 34% (w/v) sucrose and grown with shaking at 30 °C for 3-4 days. Difco nutrient broth contained 3 g l⁻¹ beef extract and 5 g l⁻¹ peptone in water to which 340 g l⁻¹ sucrose was added.

*Streptomyces* cultures were grown at 30 °C. Liquid cultures were shaken vigorously on an orbital shaker at 200 rpm, in a baffled flask when available. The *E. coli* DH5α cultures were grown at 37 °C, and all liquid cultures were shaken at 250 rpm in a New Brunswick Scientific Co. Series 25 Incubator Shaker.

*Streptomyces* spore suspension.

Spores were harvested from plate cultures of *Streptomyces* according to a modified version of the procedure of Hopwood et al. (1985). All solutions and apparati were sterilized by autoclaving prior to use. Water, typically 9 ml, was added to a well-sporulating
culture on a plate (2-3 ml for slant cultures). The agar surface was scraped with a sterile loop to suspend the spores. The liquid was then removed with a pipette and transferred to a test tube. The suspension was mixed vigorously on a vortex mixer for 1-2 minutes (min), then filtered in vacuo through fiberglass wool to remove mycelial fragments. The filtrate was centrifuged for 10 min at 1800 x g in a Sorvall H1000B rotor. The supernatant was immediately poured off. After resuspending the pellet in the remaining drop (approximately 0.5 ml), 1 ml of water was added, mixed well, and the mixture transferred to a screw-top vial containing 0.5 ml 80% (v/v) glycerol to achieve a final concentration of 20% (v/v) glycerol. The suspension was mixed and stored at -20 °C.

Harvesting of bacterial cultures.

Cells for total DNA isolation were harvested by vacuum filtration using a modified version of the method of Hopwood et al. (1985). Cells were first grown in 500 ml of nutrient broth with 34% (w/v) sucrose. The mycelium was then collected by filtration in a Buchner funnel containing two sheets of Whatman No. 50 filter paper. The mycelium was washed with 100 ml of 10% (v/v) glycerol per 500 ml of culture, and the paste then transferred to a sterile conical tube using a sterile spatula. The cell paste was stored at -20 °C or used immediately.
Preparation of competent cells for transformation.

*Escherichia coli* DH5α competent cells were produced according to a slight modification of the procedure of Dagert and Ehrich (1979). A single colony of *E. coli* DH5α was picked from an LB agar plate with a sterile inoculating loop and used to inoculate 5 ml of sterile LB broth. The bacterial culture was grown overnight at 37 °C with shaking at 250 rpm. A sterile flask containing 50 ml of LB Broth was then inoculated with 500 µl of the overnight culture and grown, typically 2-3 hours (h) at 37 °C with shaking at 250 rpm until an absorbance reading at 650 nm reached 0.2. A loopful of the culture was then streaked onto an LB plate which was incubated overnight at 37 °C to ensure purity. The remainder of the cell culture was then transferred aseptically to a 50 ml disposable, conical tube and chilled on ice for 10 min.

The cells were harvested by centrifugation in a Sorvall RT6000B Refrigerated Centrifuge for 10 min at 4 °C in a Sorvall H1000B rotor at 1800 x g. The supernatant was poured off, and the pellet was resuspended in 20 ml of cold (0-4 °C) 0.1 M CaCl₂. Cells were again pelleted by centrifugation at 1800 x g for 10 min. After the supernatant was poured off, cells were resuspended in 20 ml of cold (0-4 °C) 0.1 M CaCl₂, and then stored overnight on ice in a 4 °C refrigerator. Next, cells were harvested by centrifugation at 833 x g for 15 min. After pouring off the
supernatant, cells were resuspended in 812.5 µl of 0.1M CaCl₂ and 187.5 µl of sterile 80% (v/v) glycerol to achieve a final concentration of 15% (v/v) glycerol. Aliquots of 200 µl were distributed to 1.5 ml microcentrifuge tubes and either kept on ice for use within a few hours or stored at -80 °C for up to one month.

Agarose minigel electrophoresis.

All DNA fragments, including those from PCR, rapid plasmid preparations, chromosomal DNA isolations, and restriction enzyme digestions, were analyzed by agarose gel electrophoresis. For DNA fragments longer than 1 kbp, the gel was prepared by addition of agarose to 0.8% (w/v) in TAE buffer (40 mM Tris-Acetate and 1 mM EDTA, pH 8.0). A 1.0% (w/v) agarose gel was used for analysis of DNA fragments shorter than 1 kbp. The components were swirled to mix, weighed, and then brought to a boil in a microwave oven. Once all the agarose was in solution, the mixture was allowed to cool briefly and then weighed again. Distilled H₂O was added to replace the fluid lost by boiling. The gel mix was poured into a gel tray containing a comb and allowed to cool until solidified. The comb was then removed.

The gel tray containing the gel was placed in an electrophoresis unit containing TAE buffer. DNA samples were prepared for loading on the gel by mixing predetermined volumes of DNA and 5X sample loading buffer (25% v/v
glycerol, 0.5% w/v SDS, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol, and 50.0 mM EDTA) and then adding the mixture to the desired well. For DNA samples in the 2.0 kbp to 5 kbp range, gels were typically electrophoresed for 3 h at 52V. For DNA samples shorter than 2 kbp, gels were electrophoresed for approximately 1 h and 30 min at 52V. The DNA was stained by placing the gel in an ethidium bromide solution (0.5 μg/ml) for 30 min followed by 3 min of destaining in ddH₂O to remove residual ethidium bromide from the gel. The gel was then observed and photographed over UV radiation using a Fotodyne Transilluminator and Polaroid MP-4 camera. Polaroid type 55 film was used in order to generate positives as well as negatives, and exposure and development times were typically 45 seconds (s) and 25 s, respectively. All negatives were rinsed for 10 min under tap water before being air dried.

Isolation of chromosomal DNA.

Isolation of chromosomal DNA from Streptomyces was done according to "Procedure 1" of Hopwood et al. (1985). Mycelium (1 g wet weight for S. griseus) was suspended in 5 ml of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and lysozyme was added to a final concentration of 2 mg ml⁻¹. After swirling to mix, the sample was incubated at 30 °C, typically for 1.5-2 h. A drop of sample was removed and mixed with a drop of 10% (w/v) SDS on a microscope slide.
This was repeated until the drop cleared completely when tested in this manner. At this time, 0.5 M EDTA was added to give a concentration of 0.1 M, the solution was mixed gently by inversion, and pronase stock solution (10 mg ml\(^{-1}\)) was added to give a concentration of 0.2 mg ml\(^{-1}\). The sample was incubated at 30 °C for 5 min. Next, 10% SDS solution was added to 1% concentration, and the mixture was incubated at 37 °C for 2 h.

Cell debris was removed by phenol and chloroform extraction. While wearing gloves and eye protection, 6 ml of phenol was added, and the sample was mixed by hand for 10 min. This was followed by addition of 6 ml of chloroform. After shaking by hand for 5 min, the sample was centrifuged for 10 min at 1800 x g in a Sorvall H1000B rotor. The upper aqueous phase was carefully transferred to a fresh conical tube using a Pasteur pipette. To the phenol phase, 5 ml of TNE buffer (10 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA, pH 8.0) was added. The sample was shaken by hand for 5 min and then centrifuged as before. The upper phase was removed and pooled with the previously removed sample. The pooled phases were again extracted with phenol and chloroform as above. After centrifugation, the aqueous phase was removed to a fresh conical tube and twice extracted with 6 ml of chloroform for 5 min to remove phenol. Each time, the sample was centrifuged for 5 min, and the upper aqueous phase transferred to a fresh conical tube. For the last
transfer, a pre-weighed tube was used. The weight of the sample was determined, and RNase was added to 40 μg g⁻¹. The sample was incubated at 37 °C for 1 h.

A 0.25 volume of 5 M NaCl was added. After mixing the sample well by inversion, the DNA was precipitated by addition of 30% (w/v) PEG 8000 to 10% final concentration. The sample was mixed gently by inversion until a precipitate was visible. The DNA was spooled on a sealed Pasteur pipette and transferred to a fresh conical tube, taking care to transfer as little PEG as possible. The DNA was dissolved in 5 ml of TE buffer overnight at 4 °C.

The DNA was then precipitated using 0.6 ml of 3 M sodium acetate and 12 ml of cold absolute ethanol. The DNA was spooled onto a sealed Pasteur pipette, dipped in 2 ml of 70% (v/v) ethanol to wash, and dissolved in 1 ml of sterile TE buffer (1-2 ml additional buffer necessary for some samples to dissolve). To ensure the presence of chromosomal DNA, all isolates were analyzed by agarose gel electrophoresis. The sample was stored at 4 °C.

Design of oligonucleotides for polymerase chain reaction.

Oligonucleotide primers for polymerase chain reaction (PCR) were designed for determination of pyrimidine genes in Streptomyces. Amino acid sequence alignments were prepared from previously described pyrimidine genes in other bacteria using ClustalW 1.7 Multiple Sequence Alignment (Thompson et
al., 1994). For each gene of interest, protein sequences from 8-12 organisms were typically analyzed. Conserved regions were identified within each gene. Of these regions, those containing highly-conserved sequences of 8-10 amino acids throughout the range of bacteria sampled were selected for development of PCR oligonucleotide primers.

The DNA sequence was obtained for each of these regions, and codon usage was compared between organisms for each amino acid in the region of interest. For amino acids within this region which had very different codon usage among the organisms, those codons used most frequently by high G+C organisms (i.e., Mycobacterium) were given preference. A consensus DNA sequence was constructed using this information. This sequence was then optimized to the codon usage table for Streptomyces spp. (Mahadevan, 1995). Oligonucleotide sequences of between 20-25 bases in length were designed from this consensus sequence with preferably between 4 to 16-fold degeneracy, but no more than 32-fold. In order to ensure compatibility between primers, oligonucleotide sequences were selected with an average G+C content of 70% and melting temperature (Tm) of 69 °C (Tm’s were calculated using the OligoCalculator of Integrated DNA Technologies, Inc. which can be obtained at http://www.idtdna.com).

Two primers were designed for each gene of interest. A forward primer matched the coding strand, and a reverse
primer was prepared for a region downstream in the gene sequence on the complementary strand. To provide a standard reference, primer locations are described in relation to *Mycobacterium tuberculosis* protein sequences (These sequence data were produced by the *M. tuberculosis* sequencing group at the Sanger Centre and can be obtained from ftp://ftp.sanger.ac.uk/pub/tb/sequences). All oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The primer sequences and locations are detailed in Table 2.

**Polymerase chain reaction.**

Two types of PCR methods were utilized. For products of expected length of less than 1000 bp, Taq DNA polymerase was used with elongation times of 30 s to 1 min at 72 °C. If the product had an expected length of greater than 1 kbp, recombinant *Thermus thermophilus* (rTth) DNA polymerase was employed for longer polymerization times of generally 30 s to 1 min per kbp of product at temperatures ranging from 64 °C to 68 °C. General conditions for PCR of the shorter products, including primer annealing temperature, MgCl₂ concentration, and DNA template concentration, were optimized using *M. tuberculosis* cosmid DNA containing the *pyrB* gene and *S. griseus* 10137 chromosomal DNA with the SgB For and SgB Rev primers (Table 2). The longer extension PCR (greater than 1 kbp) conditions were optimized using the
Table 2. Oligonucleotide primers for PCR. Standard three letter codes are used for amino acids, and the following single letter codes for bases: adenosine (A), cytidine (C), guanosine (G), thymidine (T), cytidine or guanosine (S), and adenosine or thymidine (W). Number of amino acid (AA) refers to the start of the conserved region in the M. tuberculosis polypeptide. Primer names ending in "For" indicate forward primers and those ending in "Rev" are reverse primers. SgR, SgB, SgC, SgcarA, SgcarB, and SgF refer to Streptomyces griseus pyrR, pyrB, pyrC, carA, carB, and pyrF genes, respectively.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Name</th>
<th>AA#</th>
<th>Conserved AA Sequence</th>
<th>Primer Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyrR</td>
<td>SgR For</td>
<td>115</td>
<td>Val-Ile-Leu-Val-Asp-Asp-Val-Leu</td>
<td>GTS ATC GTS GTS GAC GAC GTS CT</td>
</tr>
<tr>
<td></td>
<td>SgR Rev</td>
<td>151</td>
<td>Asp-Arg-Gly-His-Arg-Glu-Leu</td>
<td>AG CTC SCC GTS SCC SCC SCC GTC</td>
</tr>
<tr>
<td>pyrB</td>
<td>SgB For</td>
<td>51</td>
<td>Phe-Phe-Glu-Asn-Ser-Thr-Arg-Thr</td>
<td>TTC TWC GAG AAC TCS ACC CGS ACC</td>
</tr>
<tr>
<td></td>
<td>SgB Rev</td>
<td>140</td>
<td>His-Pro-Thr-Gln-Ala-Leu-Leu-Asp</td>
<td>GTC CAG CAG SGC CTG SGT SGG G</td>
</tr>
<tr>
<td>pyrC</td>
<td>SgC For</td>
<td>55</td>
<td>Asp-Leu-His-Thr-His-Leu-Arg-Glu-Pro</td>
<td>GAC GTS CAC ACS CAC CTS CGS GAG C</td>
</tr>
<tr>
<td></td>
<td>SgC Rev</td>
<td>302</td>
<td>Ala-Thr-Asp-His-Ala-Pro-His</td>
<td>GTG SGG SGC GTG GTGC SGT SG</td>
</tr>
<tr>
<td>carA</td>
<td>SgcarA For</td>
<td>229</td>
<td>Leu-Ser-Asn-Gly-Pro-Gly-Asp-Pro</td>
<td>CTS TCS AAC GGS CCS GGS GAC CC</td>
</tr>
<tr>
<td></td>
<td>SgcarA Rev</td>
<td>346</td>
<td>Ser-Val-Gln-Tyr-His-Pro-Glu-Ala</td>
<td>GC CTC SGG GTG GTC CTG SAC SG</td>
</tr>
<tr>
<td>carB</td>
<td>SgcarB For</td>
<td>22</td>
<td>Gln-Ala-Cys-Glu-Phe-Asp-Tyr-Ser-Gly</td>
<td>AG GCS TGC GAG TTC GAC TAC TCS GG</td>
</tr>
<tr>
<td></td>
<td>SgcarB Rev</td>
<td>622</td>
<td>Asp-Pro-Glu-Thr-Val-Ser-Thr-Asp-Tyr</td>
<td>A GTC SGT SGA SAC SGT CTC SGG GTT</td>
</tr>
<tr>
<td>pyrF</td>
<td>SgF For</td>
<td>59</td>
<td>Lys-Pro-Glu-Val-Ala-Phe-Phe-Glu</td>
<td>AAG CCS CAG GTS GCS TTC TTC GAG</td>
</tr>
<tr>
<td></td>
<td>SgF Rev</td>
<td>93</td>
<td>Asp-Ala-Lys-Arg-Gly-Asp-Ile-Gly</td>
<td>CC GAT CTC SCC SGG CTT SCC GTC</td>
</tr>
</tbody>
</table>
control DNA provided by the manufacturer. The *S. griseus* chromosomal DNA concentration was calculated by measuring absorbance in a Beckman DU-40 spectrophotometer at a wavelength of 260 nm where an absorbance of 1.0 = 50 μg ml⁻¹.

The Taq DNA polymerase was contained in the premixed, double-concentrated PCR Master solution (Boehringer Mannheim, Indianapolis, IN). During sample preparation, all reagents and reaction mixes were stored on ice. For a 50 μl total reaction volume placed in a thin-walled, 0.6 ml PCR tube, 25 μl of PCR Master mix was added. This gives the final reaction mix 1.25 units (U) of Taq DNA polymerase in 0.005% (v/v) Brij 35, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM MgCl₂. Along with 2.5 μl of dimethyl sulfoxide (DMSO), an additional 0.6 μl of 25 mM MgCl₂ was added resulting in overall concentrations of 5% DMSO and 1.8 mM MgCl₂. Appropriate volumes of forward and reverse oligonucleotide primers were added to give 60 ng of each. Between 100-300 ng of template DNA were included in the reaction mix using cut micropipette tips to minimize DNA shearing. The final reaction volume was achieved by addition of water to give a total of 50 μl. The sample was layered with an equal volume of sterile mineral oil to minimize evaporation.

The PCR was performed on an MJ Research PTC-100 Thermal Programmable Controller (MJ Research, Inc., Watertown, MA). Initial denaturation was for 7 min at 95 °C. This was
followed by primer annealing for 1 min at 52 °C to 60 °C depending on the primers used. Elongation proceeded for 30 s to 1 min at 72 °C. The DNA was again denatured at 95 °C for 2 min. The sample was cycled through the annealing, elongation, and denaturation phases for a total of 30 times. After completion of these steps, an additional elongation period of 5 min was performed. The samples were subsequently stored at 4 °C.

The longer PCR products (greater than 1 kbp) were obtained using rTth DNA polymerase from the GeneAmp XL PCR Kit (Perkin-Elmer Applied Biosystems, Foster City, CA). The enzyme mix also contained Vent DNA polymerase. During sample preparation, all reagents and reaction mixes were stored on ice. For a 50 μl total reaction volume placed in a thin-walled, 0.6 ml PCR tube, 15 μl of 3.3X XL Buffer II was added. Next, magnesium acetate \([\text{Mg(OAc)}_2]\) was added to a final concentration of 1.2 mM. A 10 mM dNTP blend was included to give a concentration of 2.5 mM each of dATP, dCTP, dGTP, and dTTP. Appropriate volumes of forward and reverse oligonucleotide primers were added to give 60 ng of each. Using cut micropipette tips to minimize DNA shearing, between 100-300 ng of template DNA were included in the reaction mix. The last component included was 2 U of rTth DNA polymerase, XL. Water was added to bring the total reaction volume to 50 μl. The sample was layered with an equal volume of sterile mineral oil to minimize evaporation.
The PCR was performed on an MJ Research PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, MA). Initial denaturation was for 2.5 min at 94 °C. This was followed by primer annealing and elongation for 30 s to 1 min per kbp of the expected product at 64 °C to 68 °C depending on the primers used. The DNA was again denatured at 94 °C for 1 min 15 s. The sample was cycled through these annealing, elongation, and denaturation phases for a total of 17 times. A second set of conditions was then applied. After denaturation at 94 °C for 1 min 15 s, annealing and elongation proceeded for 40 s to 1 min 10 s per kbp of the product. This was repeated for a total of 13 cycles. After completion, an additional elongation period of 10 min was performed at 72 °C. The samples were subsequently stored at 4 °C.

All products were analyzed by agarose gel electrophoresis. For PCR products longer than 1 kbp, the gel was prepared by addition of agarose to 0.8% (w/v) in TAE buffer. A 1.0% (w/v) agarose gel was used for analysis of PCR products shorter than 1 kbp. The products were typically prepared for loading on the gel by mixing 12 µl with 3 µl of 5X sample loading buffer.

**Purification of PCR products.**

Immediately following separation of PCR products by agarose gel electrophoresis, DNA bands of interest were
purified using a slight modification of Procedure A of the Sephaglas BandPrep Kit (Pharmacia Biotech, Inc., Piscataway, NJ). A sterilized razor blade was used in order to excise the slice of agarose containing the DNA band of interest. Care was taken to cut as close to the DNA band as possible. The excised agarose was placed in a pre-weighed 1.5 ml microcentrifuge tube, and the weight of the agarose slice was determined by weighing the tube containing the agarose and then subtracting the weight of the empty tube. If the agarose slice weighed 250 mg or less, the following steps were carried out exactly as described. For agarose slices weighing more than 250 mg, reagent volumes were increased proportionately.

In order to dissolve the agarose slice, 250 μl of Gel Solubilizer (buffered solution containing sodium iodide) was added to the tube followed by vigorous vortexing and incubation in a 60 °C water bath for approximately 10 min or until the agarose slice had completely dissolved. Following mixing of the Sephaglas BP (20% w/v Sephaglas BP glass matrix suspended in distilled water containing 0.15% Kathon CG/ICP Biocide) container, 5 μl of the suspension was added to the dissolved gel. After an initial gentle mixing on a vortex mixer, the dissolved gel was incubated at room temperature for 5 min with gentle mixing every minute to resuspend the Sephaglas. A 30 s pulse spin at 10,000 x g in a Savant Speed Fuge HSC10K microcentrifuge was employed to
pellet the Sephaglas and adhered DNA. The supernatant was carefully removed and discarded without disturbing the Sephaglas pellet. The 30 s pulse spin was repeated, and any remaining supernatant was again carefully removed and discarded.

The pellet was then resuspended in 80 μl of Wash Buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA and 0.1 mM NaCl to which 18 ml of absolute ethanol was initially added) by pipetting up and down several times, followed by a 30 s pulse spin and careful removal of the supernatant. This step was repeated twice for a total of three washes. After the third wash and removal of supernatant, the tube was tapped to partially disperse the Sephaglas pellet and the top of the tube was opened. The tube was inverted and placed on a paper towel on the bench top at room temperature for 45 min or until the pellet was completely dry. The pellet was then resuspended in 15-20 μl of sterile ddH₂O by pipetting gently up and down periodically for a total of 8 min at room temperature. The tube was centrifuged at 10,000 x g for 1 min, and the supernatant was removed and placed in a clean microcentrifuge tube without disturbing the Sephaglas pellet. The eluted DNA was stored on ice for all subsequent steps.

The eluted DNA was immediately analyzed by agarose gel electrophoresis in order to estimate concentration and confirm size and purity of the DNA sample. For DNA bands
longer than 1 kbp, the gel was prepared by addition of agarose to 0.8% (w/v) in TAE buffer. A 1.0% (w/v) agarose gel was used for analysis of DNA bands shorter than 1 kbp. The gel tray containing the gel was placed in an electrophoresis unit containing TAE buffer. Eluted DNA bands were prepared for loading on the gel by mixing 4 μl with 1 μl of 5X sample loading buffer.

Cloning of purified PCR products.

Cloning of purified PCR products was achieved using the TA Cloning Kit Dual Promoter (Invitrogen, Carlsbad, CA). In order to avoid degradation of the single 3' A-overhangs on the PCR products and resulting reduction in ligation efficiency, ligations were always set up on the same day that PCR products were generated. Typically 4-5 μl (approximately 50-100 ng) of the eluted PCR product was added to a 0.6 ml, thin walled PCR tube on ice. Next, 1 μl of 10X Ligation Buffer (60 mM Tris-HCl, pH 7.5, 60 mM MgCl₂, 50 mM NaCl, 1 mg ml⁻¹ bovine serum albumin, 70 mM β-mercaptoethanol, 1 mM ATP, 20 mM dithiothreitol, 10 mM spermidine), 2 μl of pCRII vector (25 ng μl⁻¹), and sterile water to a total volume of 9 μl were included in the PCR tube. The addition of 1 μl of T4 DNA Ligase (4.0 Weiss units) brought the total ligation reaction volume to 10 μl. The ligation reaction was incubated at 14 °C for 16 h in an MJ Research PTC-100 Thermal Programmable Controller.
Transformation was achieved by adding the entire 10 µl ligation reaction into 200 µl of competent *E. coli* DH5α in a 1.5 ml microcentrifuge tube. The mixture was incubated on ice for 10 min and then heat shocked in a 42 °C water bath for 2 min. The mixture was then diluted with 1 ml of LB broth and incubated for 1 h with shaking at 250 rpm at 37 °C. During this incubation period, 50 µl of 2% 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) and 10 µl of 100 mM isopropylthiogalactoside (IPTG) were added to three LB agar + Ampicillin (Amp, 50 µg ml⁻¹) plates per ligation with a sterile spreader. These LB plates were then placed in a 37 °C incubator for 30 min to allow the added reagents to dry.

Following the 1 h incubation, 100 µl and 50 µl of the transformed cells were plated onto two different LB + Amp + X-gal + IPTG plates using a sterile spreader. The remaining transformed cells were pelleted by centrifugation at 10,000 x g for 4-5 s. The supernatant was poured off and the pellet was resuspended in the remaining drop by brief mixing with a vortex mixer. The resuspended cells were then plated onto the remaining LB + Amp + X-gal + IPTG plate. These plates were then incubated at 37 °C for 16 h.

An LB agar master plate containing Amp (50 µg ml⁻¹), 0.004% X-gal, and 0.04 mM IPTG was made by picking 49 different white colonies (containing *E. coli* DH5α with pCRII vector + insert) with sterile toothpicks and inoculating the
master plate using a 50 square grid. The pCRII vector contained an ampicillin resistance gene, thus allowing transformed cells to grow in ampicillin. One blue colony (containing *E. coli* DH5α with pCRII vector only) was selected to serve as a control. The master plate was incubated overnight at 37°C. Fifteen white colonies and one blue colony were selected from the master plate and inoculated into 5 ml LB broth containing Amp (50 µg ml⁻¹) by using sterile toothpicks. The transformed cells were incubated 16 h at 37°C with shaking at 250 rpm.

**Isolation of plasmid DNA by alkaline lysis.**

Plasmid DNA was isolated according to the alkaline lysis method (Birnboim & Doly, 1979). After incubation of transformed cells overnight at 37°C with shaking at 250 rpm, 1.5 ml of the 5 ml culture was transferred to a sterile 1.5 ml microcentrifuge tube. The 1.5 ml culture was centrifuged for 1 min at 10,000 x g in a Savant Speed Fuge HSC10K microcentrifuge while the remainder of the overnight culture was stored at 4°C. The supernatant was poured off and the bacterial pellet resuspended by vortexing in 100 µl of ice-cold solution A (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0, and 6.0 mg ml⁻¹ lysozyme). The tube was left at room temperature for 5 min with the top of the tube open. Next, 200 µl of freshly prepared solution B (0.2 N NaOH and 1.0% SDS) was added, and the contents of the tube
was mixed by rapid inversion two or three times. The tube was then stored on ice for 5 min, followed by the addition of 150 µl of an ice-cold solution of potassium acetate, pH 4.8 (3 M potassium and 5 M acetate). A stock solution of potassium acetate was made up beforehand by combining 60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, and 28.5 ml ddH₂O. The contents of the tube were mixed by rapid inversion two or three times, and the tube was stored on ice for 5 min. In order to pellet the cell debris and most of the chromosomal DNA, the tube was centrifuged for 5 min at 10,000 x g at 4 °C, and the supernatant was transferred to a fresh, 1.5 ml microcentrifuge tube.

Contaminating proteins and lipids were removed by phenol:chloroform extraction. An equal volume (usually 450 µl) of phenol:chloroform (1 volume water saturated phenol and 1 volume chloroform) was added and mixed by vortexing. The tube was centrifuged at 10,000 x g for 2 min and the supernatant (upper aqueous layer) carefully transferred to a fresh, 1.5 ml microcentrifuge tube. Two volumes (usually 750 µl) of -20 °C 100% ethanol (EtOH) were added at room temperature, mixed by vortexing, and allowed to stand at room temperature for 2 min. Next, in order to pellet the plasmid DNA, the tube was centrifuged at 10,000 x g for 5 min at room temperature, and the supernatant was removed. The tube, with the top open, was placed in an
inverted position on a paper towel for approximately 30 min to allow the EtOH to evaporate.

To remove residual salts, 1 ml of -20 °C 70% (v/v) EtOH was added, and the tube was centrifuged for 5 min at 10,000 × g at room temperature. The supernatant was removed using a siliconized, drawn-out Pasteur pipette, and the pellet was dried for approximately 3 min in a Savant Speed Vac Concentrator. The plasmid DNA pellet was resuspended in 28 μl of ddH₂O with two freeze-thaw cycles consisting of incubation of the tube at -78.5 °C in solid carbon dioxide (CO₂) for 10 min followed by a 5 min incubation in a 65 °C water bath. Following each 65 °C incubation, the plasmid DNA and ddH₂O were mixed with a vortex mixer and centrifuged at 10,000 × g for 4 s to collect the mixture at the bottom of the tube. To degrade any RNA present in the sample, 2 μl of RNase A, pre-heated for 5 min in a 65 °C water bath, was added and mixed briefly on a vortex mixer. After a brief centrifugation to collect the tube contents at the bottom of the tube, the tube was placed in a 37 °C water bath for 30 min. All plasmid DNA was analyzed by agarose minigel electrophoresis by loading 4 μl of plasmid DNA combined with 1 μl of 5X Loading Buffer on a 0.8% agarose gel. The agarose gel was placed in 1X TAE and electrophoresed at 52V for approximately 2 h. The remaining 26 μl of plasmid DNA was stored at -20 °C.
Analysis of plasmid DNA by restriction enzyme digestion.

To determine which plasmids had inserts of the desired size, a restriction enzyme digestion was employed in order to remove the insert from the pCRII vector. The restriction enzyme, EcoRI (New England Biolabs, Beverly, MA), was chosen based on the fact that it has only two cut sites in the pCRII vector which reside on either side of the insert. In addition, the likelihood of encountering an EcoRI site, GAATTC, within Streptomyces griseus is very small due to its high G+C content. Therefore, EcoRI digestions of plasmid DNA should produce only two bands, 3.9 kbp for the vector and the desired length for the insert, when separated by agarose gel electrophoresis.

To set up a restriction enzyme digest, the following contents were added to a 1.5 ml microcentrifuge tube: 1-5 μl of plasmid DNA isolated by alkaline lysis, 1 μl of EcoRI restriction enzyme, 3 μl of 10X EcoRI buffer (50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl₂, 0.025% Triton X-100, pH 7.5 @ 25 °C), and ddH₂O up to a total volume of 30 μl. The contents were then incubated in a 37 °C water bath for 2 h and 30 min, followed by heat inactivation of the EcoRI in a 65 °C water bath for 20 min. The EcoRI digest was then analyzed by loading a combination of 12 μl of the EcoRI digest and 3 μl of 5X Loading Buffer on a 0.8% agarose gel placed in 1X TAE. The sample was electrophoresed at 52V for approximately 2 h.
Preparation of plasmid DNA for sequencing.

After analyzing EcoRI digested plasmid DNA products, those pCRII vectors believed to contain inserts of the appropriate size were further purified for double stranded (ds) DNA sequencing. Of the remaining 3.5 ml of the desired overnight culture previously used for isolation of plasmid DNA by alkaline lysis, 500 µl was removed and used to inoculate 50 ml of LB broth containing freshly added Ampicillin (50 µg ml⁻¹). This culture was incubated for 16-20 h at 37 °C with shaking at 250 rpm.

Plasmid DNA was purified from this culture using the RPM Spin Midi Kit and protocol (BIO 101, Inc., La Jolla, CA). Specific reagents included in each solution were not available from BIO 101. The 50 ml culture was transferred to a 50 ml disposable conical vial and centrifuged at 1800 x g in a Sorvall RT6000B Refrigerated Centrifuge using an H1000B Rotor at 4 °C for 5 min. After pouring off the supernatant, the cell pellet was resuspended in 500 µl ddH₂O by vortexing and subsequently transferred to a 2 ml microcentrifuge tube. The 2 ml tube was then centrifuged for 20 s at 10,000 x g, and the supernatant was decanted. At this stage, the cell pellet was either stored at -20 °C until ready to process or used immediately. Those pellets that were stored at -20 °C were first thawed for 8 min at 37 °C before continuing with the following plasmid purification.
Plasmid DNA isolation and purification began with the addition of 200 μl of Pre-Lysis Solution. The bacterial pellet was completely resuspended in this solution by mixing on a vortex mixer. Next, 400 μl of Alkaline Lysis Solution was added, and the tube was inverted gently 15 times. Following this, 300 μl of ice-cold Neutralizing Solution was added, and the tube was shaken vigorously 3-5 times until a uniform white precipitate formed. The tube was then centrifuged for 5 min at 10,000 x g, and the supernatant was carefully transferred to a new 2 ml microcentrifuge tube.

GLASSMILK Spin Buffer containing a silica matrix was incubated for 10 min in a 65 °C water bath, and then 900 μl of this Spin Buffer was added. The tube was inverted gently for 5 min at room temperature and then centrifuged for 5 s at 10,000 x g at room temperature. The supernatant was decanted, leaving the plasmid DNA bound to the GLASSMILK, and 500 μl of Wash Solution containing EtOH was added. The GLASSMILK/plasmid DNA complex was resuspended by gentle pipetting up and down, and the solution was then transferred to a kit-supplied SPIN Filter. This filter was centrifuged for 20 s at 10,000 x g to separate the GLASSMILK/plasmid DNA complex from the Wash Solution. The Catch Tube containing the Wash Solution was emptied and the Spin Filter reassembled. A second wash was performed by adding an additional 500 μl of Wash solution and recentrifuging for 5 min at 10,000 x g to dry the
GLASSMILK/plasmid DNA complex trapped by the Spin Filter. The Spin Filter was then transferred to a new kit-supplied Catch Tube, and 100 μl of Elution Solution containing Tris-HCl and EDTA was added to remove the plasmid DNA from the GLASSMILK. The plasmid DNA was eluted by gently stirring and pipetting the solution up and down with a 200 μl pipette until the GLASSMILK/plasmid DNA complex was completely resuspended. The Catch Tube was then centrifuged at 10,000 x g for 5 min to collect the eluted DNA in the Catch Tube. The Spin Filter containing the GLASSMILK was discarded. The eluted plasmid DNA was analyzed by agarose minigel electrophoresis by loading a combination of 1-4 μl of the eluted plasmid DNA with the appropriate volume of 5X Loading Buffer on a 0.8% agarose gel in 1X TAE.

To confirm that the eluted plasmid DNA had the pCRII vector and appropriate insert size, all samples were analyzed by EcoRI digestion in the same manner as previously described.

**Double stranded DNA sequencing.**

Those PCR products that had been ligated into pCRII vector, transformed into E. coli DH5α, and isolated and purified by both alkaline lysis and by Spin Midi Prep, were then analyzed by double stranded DNA sequencing using the dideoxyribonucleotide method of chain termination (Sanger et al., 1977). Sequencing reactions were set up according to a
slight modification of the protocol provided for the T7 Sequenase version 2.0 DNA sequencing kit (Amersham Life Science, Inc., Cleveland, OH). For each plasmid to be sequenced, both M13 Forward (-40) and M13 Reverse primers were utilized, thus allowing sequence to be read from opposite ends of the insert. Approximately 5 µg of plasmid DNA was placed into a 1.5 ml microcentrifuge tube, and ddH₂O was added to achieve a total volume of 30 µl. To denature the DNA, 3 µl of freshly prepared 2 N NaOH was added, and the mixture was incubated at room temperature for 5 min. Next, 120 µl of -20 °C 100% EtOH was added along with 5 µl of 3 M sodium acetate, pH 5.0. This was mixed and incubated at -78.5 °C in solid CO₂ for 5 min. This was then centrifuged at 10,000 x g for 20 min at 4 °C. The supernatant was removed without disturbing the pellet using a siliconized, drawn-out Pasteur pipette. To remove residual salts, 200 µl of -20 °C 70% (v/v) EtOH was added, and the tube was centrifuged for 5 min at 10,000 x g at 4 °C. The supernatant was again removed with a siliconized, drawn-out Pasteur pipette. This 70% (v/v) EtOH wash was repeated, and the pellet was dried for 3-5 min in a Savant Speed Vac Concentrator. The pellet was resuspended in 7 µl ddH₂O with two freeze-thaw cycles consisting of incubation of the tube at -78.5 °C in solid CO₂ for 10 min followed by a 5 min incubation in a 65 °C water bath. Following each 65 °C incubation, the plasmid DNA and ddH₂O were mixed with a vortex mixer and centrifuged at
10,000 xg for 4 s to collect the mixture at the bottom of the tube.

Following this initial denaturation and cleanup, 2 μl of T7 Sequenase Reaction Buffer (200 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, and 250 mM NaCl) from the T7 Sequenase Kit was added along with 1 μl of either M13 Forward (-40) or M13 Reverse primers (0.5 pmol μl⁻¹). Incubation at 37 °C for 45 min allowed the primer to anneal to the template. After placing this tube on ice, four 0.5 ml tubes were labeled and filled with 2.5 μl of the appropriate termination mix (80 μM dGTP, 80 μM dATP, 80 μM dCTP, 80 μM dTTP, 50 mM NaCl, and 8 μM of the appropriate ddNTP) from the red-capped tubes from the T7 Sequenase Kit. The termination tubes were stored on ice until use. In another 0.5 ml tube, a 1:5 diluted labeling mix was prepared, usually by adding 2 μl of the T7 Sequenase dGTP Labeling Mix (7.5 μM dGTP, 7.5 μM dCTP, and 7.5 μM dTTP) from the green-capped tube to 8 μl of ddH₂O. The 1:5 Labeling Mix was stored on ice. Diluted T7 Sequenase DNA polymerase was prepared in a separate 0.5 ml tube by adding the following components on ice, all from the T7 Sequenase Kit: 1.2 μl of inorganic pyrophosphatase (5 U ml⁻¹ in 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 50% v/v glycerol), 7.2 μl of glycerol enzyme dilution buffer (20 mM Tris-HCl, pH 7.5, 2 mM DTT, 0.1 mM EDTA, and 50% v/v glycerol), and 1.2 μl of T7 Sequenase version 2.0 DNA
polymerase (13 U μl⁻¹ in 20 mM KPO₄, pH 7.4, 1 mM DTT, 0.1 mM EDTA, and 50% v/v glycerol).

The four termination tubes were then pre-warmed in a 48 °C heating block. The following components were added to the ice-cold annealed DNA mixture: 1 μl of 0.1 M Dithiothreitol (DTT from the T7 Sequenase Kit), 2 μl of the 1:5 Labeling Mix, 1 μl of Dimethylsulfoxide (DMSO), 0.5 μl of Redivue [α-35S] dATP (12.5 μCi μl⁻¹), and 2 μl of diluted T7 Sequenase DNA Polymerase. This mixture was centrifuged at 10,000 x g for 3 s and then incubated at 37 °C for 2-5 min. To allow chain termination to occur, 3.5 μl of the labeling reaction mixture was added to each of the four termination tubes and incubated for 5 min at 48 °C. The high temperatures for the labeling (37 °C) and termination (48 °C) reactions were employed to help prevent the formation of secondary structure, which can cause premature termination, especially in high G+C content bacteria such as Streptomyces.

Following the termination reactions, a "chase" step preceded by boiling was performed in which DNA fragments terminated with a dNTP instead of a ddNTP were extended with a long stretch of dNTP's, thus making these fragments much longer and thereby helping to eliminate artifact banding (Redston and Kern, 1994; Fawcett & Bartlett, 1990; Fawcett & Bartlett, 1991; McCrea et al., 1993; Stupi & Brummet, 1991). The longer fragments would stay at the top of the sequencing
gel, and therefore, would not be incorrectly interpreted as true terminations. The four termination tubes were boiled for 1.5 min and then placed on ice for 15 min. During this 15 min incubation on ice, a Terminal deoxynucleotidyl transferase (TdT)/dNTP cocktail was prepared by adding 6 $\mu$l of a 10 mM dNTP blend, 1.9 $\mu$l of a 17 U $\mu$l$^{-1}$ TdT solution (United States Biochemical, Cleveland, OH), and 9 $\mu$l of Sequenase enzyme dilution buffer (10 mM Tris-HCl, pH 7.5, and 5 mM DTT) for a total volume of 16.9 $\mu$l. Following the 15 min incubation on ice, 1 $\mu$l of the TdT/dNTP cocktail was added to each termination tube, and this mixture was incubated at 48 °C for 30 min. Next, 4 $\mu$l of Stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF) from the T7 Sequenase Kit was added to each termination tube and mixed by pipetting up and down. The tubes were then centrifuged at 10,000 $\times$ g for 3 s and frozen at -20 °C.

**Preparation of polyacrylamide sequencing gels.**

A gel cassette was assembled to provide a reservoir for the sequencing gel. The sequencing plates measured 50.8 cm x 40.6 cm and were cleaned twice with glass cleaner followed by 95% (v/v) EtOH. The inside of the notched plate was siliconized with chlorinated organopolysiloxane in heptane to prevent the gel from adhering to this plate. The outside of the non-notched plate had a thermometer so that the
temperature of the gel could be regulated. After adding spacers to the long dimensions of one plate, the other gel plate was positioned on top of the plate with the spacers, and all of the sides except for the top were taped together.

All samples were analyzed on a 6% polyacrylamide sequencing gel prepared by mixing the following components in a 250 ml beaker: 42.4 g of urea, 20 ml of a 29% acrylamide/1% bis-acrylamide mixture stored at 4 °C, and 10 ml of 10X TBE sequencing buffer (890 mM Tris base, 890 mM boric acid, and 20 mM EDTA). These first three ingredients were stirred and heated until they dissolved, and then ddH₂O was added up to 100 ml. The mixture was again stirred until it dissolved completely. Undissolved particles were removed from the solution by filtering it through a Buchner funnel containing a piece of Whatman No. 50 filter paper, and the filtrate was collected in a 250 ml Erlenmeyer vacuum filtration flask. Next, 0.12 g of solid ammonium persulfate was added and the solution swirled to mix. After covering the flask with a rubber stopper, the solution was degassed in vacuo until all gasses were removed (about 3-5 min), and the contents were then carefully poured into a 250 ml beaker.

Immediately before pouring the solution into the gel cassette, 20 μl of N,N,N’,N’-Tetramethylethylenediamine (TEMED) was added, and the solution was briefly stirred with a stir bar and magnetic stirrer. The gel was then poured by
pulling up 60 ml of the solution into a 60 cc syringe and then slowly and steadily discharging the solution into one corner of the gel plates which had previously been set at a 45° angle. The gel cassette was then placed on rubber stoppers in a horizontal position, and the comb was inserted with its teeth flush with the outside edge of the plate. Clamps were placed over the two plates at the level of the comb in order to prevent movement of the comb, and Saran wrap was used to cover the open end of the plate to prevent the gel from drying out. The gel was allowed to polymerize for a minimum of 6 h (preferably overnight).

**Loading and electrophoresing the sequencing gel.**

After allowing the gel to polymerize, the shark's tooth well-forming comb and the tape holding the two plates together was removed. The plates holding the gel were then placed into the gel chamber apparatus, and 1X TBE buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA) was added to the upper and lower chambers. Air bubbles were removed from the bottom of the gel cassette, and the well-forming comb was inserted with the shark's teeth facing the top of the gel. A 60 cc syringe was used to eject 1X TBE at the top surface of the gel in order to remove any debris that was present. In order to warm up the plates to prevent renaturation of DNA, the gel was pre-electrophoresed for 1 h.
at 72 W (approximately 1600V) after adding 2 µl of Stop solution to one of the outer wells on each side of the gel.

Once the plates had reached a temperature of 50-55 °C, the power supply was turned off, and a needle and syringe was used to wash out each of the wells to be used with 1X TBE. Samples to be loaded were first denatured at 90 °C for 2 min followed by a 5 min quick cool on ice. Next, 3 µl of sample was loaded in each well with the order from left to right always being GATC. Samples were electrophoresed at 72 W (approximately 1600V) until the xylene cyanol dye had migrated a distance of 20 cm. The same samples were then reloaded, and the xylene cyanol was allowed to migrate 40 cm. The 40 cm run allowed sequence to be read from the edge of the pCR11 vector TA cloning site and into the beginning of the insert, whereas the 60 cm run allowed sequence to be read further into the insert. Longer runs were employed when necessary.

Following completion of electrophoresis, the power supply was turned off, the plates were removed from the sequencing chamber and placed on the bench top, and the siliconized, notched plate was separated from the gel using a spatula. A 43 cm x 35 cm piece of Whatman 3MM paper was placed on top of the gel, and a pipette was rolled on top of the Whatman paper to allow the gel to adhere to the Whatman paper. The Whatman paper, with gel adhered, was placed gel side up on the bench top, and the gel was covered with Saran
wrap. The gel was dried for 3 h at 76 °C in a Drygel Sr. Slab Gel Dryer Model SE 1160 supplied by Hoefer Scientific Instruments (San Francisco, CA) attached to a Savant refrigerated solvent trap.

**Autoradiography of sequencing gels.**

After the sequencing gel was dried completely, the Saran wrap was removed, and the gel adhered to Whatman paper was placed in a Fisher Biotech metal cassette with the gel side facing up. The number of counts min⁻¹ was measured using a W.B. Johnson GSM-5 Survey Meter (Mountain Lakes, NJ) in order to determine the exposure time. In a dark room, Kodak XAR-5 film was placed on top of the gel, and the cassette was closed and placed in a dark drawer to expose the film for approximately 72-120 h. The film was developed using an All Pro 100 developer.
RESULTS AND DISCUSSION

Polymerase chain reaction products.

Products were obtained by polymerase chain reaction (PCR) from *S. griseus* 10137 DNA using oligonucleotide primers corresponding to conserved regions of pyrimidine biosynthetic genes (Fig. 4-6). In each case, the *Mycobacterium tuberculosis* operon was used as a model for comparison (Fig. 7). Internal PCR products were obtained from *S. griseus* chromosomal DNA when the forward primer corresponded to a region in one pyrimidine gene and the reverse primer to a region downstream on the complementary strand within the same pyrimidine gene (Fig. 4). Internal PCR products were generated for *pyrB*, *pyrC*, *carA*, *carB*, and *pyrF*. In each case the size of the internal product was very similar to that expected for *M. tuberculosis*, indicating that these primers had indeed bound to the expected pyrimidine gene and that PCR using primer combinations between two or more pyrimidine genes could now be attempted.

Therefore, PCR was carried out using a forward primer corresponding to a region within one pyrimidine gene and a reverse primer from a pyrimidine gene believed to be located downstream from the first. In this manner, products were obtained which spanned two or more genes (Fig. 5-6).
Figure 4. Internal pyrimidine gene products from PCR of *S. griseus* 10137 DNA. Samples shown on 1.0% agarose gels.

Lane 1 - DNA Marker IV; Lane 2 - internal pyrB using SgB For and SgB Rev primers; Lane 3 - internal pyrC using SgCFor and SgC Rev primers; Lane 4 - lambda cut w/ BstEII; Lane 5 - DNA Marker IV; Lane 6 - internal carA using SgcarA For and SgcarA Rev primers; Lane 7 - DNA Marker IV; Lane 8 - internal carB using SgcarB For and SgcarB Rev primers; Lane 9 - internal pyrF using SgF For and SgF Rev primers; and Lane 10 - lambda cut w/ BstEII.
Figure 5. *S. griseus* 10137 PCR products which span two or more pyrimidine genes and originate within *pyrR*. Samples shown on 0.8% agarose gels. Lane 1 - DNA Marker IV; Lane 2 - *pyrR*-pyrB using SgR For and SgB Rev primers; Lane 3 - *pyrR*-carA using SgR For and SgcarA Rev primers; Lane 4 - GIBCO BRL 1 kb ladder; Lane 5 - GIBCO BRL 1 kb ladder; and Lane 6 - *pyrR*-pyrF using SgR For and SgF Rev primers.
Figure 6. *S. griseus* 10137 PCR products which span two or more pyrimidine genes. Samples shown on 0.8% agarose gels. Lane 1 - DNA Marker IV; Lane 2 - pyrB-pyrC using SgB For and SgC Rev primers; Lane 3 - DNA Marker IV; Lane 4 - pyrC-carA using SgC For and SgcarA Rev primers; Lane 5 - carA-carB using SgcarA For and SgcarB Rev primers; Lane 6 - carB-pyrF using SgcarB For and SgF Rev primers; Lane 7 - lambda cut w/ BstEII; Lane 8 - carA-pyrF using SgcarA For and SgF Rev primers; Lane 9 - GIBCO BRL 1 kb ladder; and Lane 10 - pyrC-carB using SgC For and SgcarB Rev primers.
Figure 7. *M. tuberculosis* pyrimidine operon with PCR primer locations and orientations (arrows). Expected products for primer combinations utilized are shown. Numbers represent base pairs in each product.
M. tuberculosis Pyrimidine Operon

- pyrR
- pyrB
- pyrC
- orf
- carA
- carB
- pyrF

Genetic map positions:
- pyrR: 128
- pyrB: 291
- pyrC: 762
- orf: 1730
- carA: 374
- carB: 1782
- pyrF: 125
- 677
- 2679
- 3579
- 4597
- 4089
- 4033
- 7748
Using the SgR For primer with the SgB Rev primer produced a pyrR-pyrB fragment of approximately 770 bp (Fig. 5). The fact that this PCR product is about 100 bp longer than that of M. tuberculosis indicates the possibility that there is no overlap between the pyrR and pyrB genes in S. griseus. A combination of the SgR For and SgcarA Rev primers resulted in a pyrR-carA fragment of approximately 4.2 kbp (Fig. 5). This again is a little over 100 bp longer than expected for M. tuberculosis. In addition, the fact that this fragment is at least as long as that expected for M. tuberculosis provides evidence that an open reading frame (ORF), as seen in M. tuberculosis, exists between pyrC and carA.

Further evidence for the existence of this ORF in S. griseus is provided by the pyrC-carA (generated using SgC For and SgcarA Rev primers) and pyrC-carB (generated using SgC For and SgcarB Rev primers) PCR products, both of which are approximately 100 bp larger than that expected for M. tuberculosis (Fig. 6). If this ORF did not exist in S. griseus, then one would expect the PCR products to be 500 bp (the approximate size of the ORF in M. tuberculosis) shorter than that expected for M. tuberculosis. Large size discrepancies were noted for the products from SgR For to SgF Rev (pyrR-pyrF), SgcarA For to SgF Rev (carA-pyrF), and SgcarB For to SgF Rev (carB-pyrF), all of which were approximately 1.2 kbp larger than expected, thus indicating
the possibility of an additional gene located between carB and pyrF (Fig. 5-6).

Using the SgB For primer with the SgC Rev primer resulted in a 1.7 kbp pyrB-pyrC product which corresponds to the same product from M. tuberculosis (Fig. 6). Likewise, the SgcarA For to the SgcarB Rev reaction produced a carA-carB PCR product of 2.3 kbp which is the same size as that expected for M. tuberculosis (Fig. 6). The PCR products obtained for S. griseus were used to construct a probable Streptomyces pyrimidine operon (Fig. 8).

**Partial sequence analysis of the pyrimidine operon in S. griseus.**

Partial sequence analysis was carried out using the Sanger dideoxy method on double-stranded plasmid DNA to verify that the PCR products obtained from S. griseus actually resided within the pyrimidine operon. An approximately 300 bp PCR product from within the pyrB gene of S. griseus was ligated into pCRII vector, transformed into E. coli DH5α, and isolated by alkaline lysis. An EcoRI digest on this plasmid produced two DNA bands, one of 3.9 kbp corresponding to the pCRII vector, and one of approximately 300 bp corresponding to a portion of the pyrB gene within S. griseus. After further purification of this plasmid through a Spin Midi Prep, the sample was ready for sequencing.
Figure 8. Probable *Streptomyces* pyrimidine operon with PCR primer locations and orientations (arrows). Actual products from *S. griseus* 10137 DNA for primer combinations utilized are shown. Numbers represent estimated base pairs in each product. Gene overlaps are hypothetical.
Probable Streptomyces Pyrimidine Operon

\[\text{pyr}R \quad \text{pyr}B \quad \text{pyr}C \quad \text{orf}1 ? \quad \text{car}A \quad \text{car}B \quad \text{pyr}D ? \quad \text{pyr}F\]

- 282
- \(\sim 750\)
- \(\sim 400\)
- \(\sim 1800\)
- \(\sim 125\)
- \(\sim 1700\)
- \(\sim 2300\)
- \(\sim 770\)
- \(\sim 2800\)
- \(\sim 4800\)
- \(\sim 4650\)
- \(\sim 5300\)
- \(\sim 4200\)
- \(\sim 8900\)
The nucleotide sequence of the entire 282 bp internal pyrB PCR product (Fig. 4) was determined, and 60 bp and the corresponding 20 amino acids (AA’s) are presented for illustration (Fig. 9). This 60 bp pyrB sequence has a 68.3% G+C content with either a G or a C occupying the third position of each codon 100% of the time. Likewise, the 770 bp pyrR-pyrB PCR product (Fig. 5) was cloned into pCRII vector and a partial nucleotide sequence was obtained. For illustration, 60 bp within the S. griseus pyrR and the corresponding 20 AA’s are presented (Fig. 11). This 60 bp pyrR sequence has an 80% G+C content with either a G or a C occupying the third position of each codon 100% of the time.

The 20 AA’s selected for illustration from the S. griseus ATCase (encoded by pyrB) and uracil phosphoribosyltransferase, or UPRTase, (encoded by pyrR) show a high degree of homology with AA’s from these same enzymes in several other known bacteria (Fig. 10 & 12). This evidence confirms that the SgR and SgB primers are indeed binding to the correct pyrimidine genes, and therefore, that the Streptomyces pyrimidine operon organization is as suggested by the PCR product sizes. The highest degree of AA homology was found with M. tuberculosis and T. aquaticus. In fact, this S. griseus pyrB AA sequence shares 80% identity and 90% homology with the M. tuberculosis pyrB and 75% identity and 85% homology with the T. aquaticus pyrB, thus indicating a close phylogenetic
Figure 9. Partial nucleotide and corresponding AA sequence of the 282 bp pyrB PCR product obtained from between the conserved carbamoylphosphate binding sites within *S. griseus*. One letter abbreviations for amino acids are as follows: Alanine (A), Aspartate (D), Glutamate (E), Glycine (G), Leucine (L), Lysine (K), Methionine (M), Serine (S), Threonine (T), and Valine (V).

5' GGC TCC TCC GTC TCC AAG GGC GAG TCC CTG AAG GAC ACC GCG
  G S S V S K G E S L K D T A

CTG ACC CTG GAG GCG ATG 3'
  L T L E A M
Figure 10. Amino acid comparison of 20 AA's from the S. griseus ATCase with several other known bacterial ATCases. All sequences, with the exception of the E. coli sequence, were obtained from references cited in Figures 2 and 3. The E. coli sequence was obtained from GenBank and is available on the Internet at http://www.ncbi.nlm.nih.gov. Amino acids in bold represent homologous residues. Asterisks denote identical residues. One letter abbreviations for amino acids are as follows: Alanine (A), Arginine (R), Aspartate (D), Glutamate (E), Glutamine (Q), Glycine (G), Isoleucine (I), Leucine (L), Lysine (K), Methionine (M), Asparagine (N), Serine (S), Threonine (T), Tyrosine (Y), and Valine (V). Residues considered to be homologous are: 1. I = L = M = V. 2. A = S = T = G. 3. K = R

Streptomyces griseus  Mycobacterium tuberculosis  Bacillus subtilis  Lactobacillus plantarum  Streptococcus pneumoniae  Pseudomonas aeruginosa  Thermus aquaticus  Escherichia coli

* * * * *

GSSVSK-GESLKDTALTLEAM  GSSVGR-GESLRTALTLLRAA  STSVQQ-GETLYDTIRTLESI  TSSVTR-GESLLTDLKTIEAI  TSSVNK-GETLYDTILTLSAL  TSSTSK-GETLTDTLRNLEAM  TSSLQK-GESEYKDTLLTLEAM  NTSLGKKGETLADTISVISTY
Figure 11. Partial nucleotide and corresponding AA sequence of the \textit{pyrR} gene of \textit{S. griseus} obtained from the 770 bp \textit{pyrR-pyrB} PCR product. One letter abbreviations for amino acids are as follows: Alanine (A), Arginine (R), Aspartate (D), Glutamate (E), Glycine (G), Isoleucine (I), Leucine (L), Lysine (K), Methionine (M), Phenylalanine (F), Proline (P), Serine (S), Threonine (T), and Valine (V).
Figure 12. Amino acid comparison of 20 AA’s from the uracil phosphoribosyltransferase (UPRTase) encoded by the pyrR gene of *S. griseus* with several other known UPRTases encoded by pyrR. All sequences, with the exception of the *P. putida* sequence, were obtained from references cited in Figures 2 and 3. The *P. putida* sequence was obtained from A. Kumar (personal communication). Amino acids in bold represent homologous residues. Asterisks denote identical residues. One letter abbreviations for amino acids are as follows: Alanine (A), Aspartate (D), Glutamate (E), Glycine (G), Leucine (L), Lysine (K), Methionine (M), Serine (S), Threonine (T), and Valine (V). Residues considered to be homologous are: 1. I = L = M = V. 2. A = S = T = G. 3. K = R. 4. F = Y. 5. D = N.

** ** ***

*Streptomyces griseus*  
*Mycobacterium tuberculosis*  
*Bacillus subtilis*  
*Lactobacillus plantarum*  
*Enterococcus faecalis*  
*Pseudomonas putida*  
*Thermus aquaticus*  
*Clostridium acetobutylicum*  

**PSGRTIRAALDALKGRPR**  
**YSGRSVRSLALRDVGRPR**  
**YTGRTVRAGMDALVDVGRPS**  
**FTGRTVAALDALMDHGRPA**  
**YTGRRTAAMDAVMMLDGRPR**  
**MSGRTIRAALNLFYGRPA**  
**YTGRRTARAALDAMILDGPR**  
**YTGRRTCAATEAMHRGRPK**
relationship with these bacteria. This similarity with *M. tuberculosis* is expected, as *M. tuberculosis*, like *Streptomyces*, is a Gram-positive bacterium with a high G+C content.

On the other hand, it is not so apparent why *T. aquaticus*, a Gram-negative bacterium, would share such high homology with *Streptomyces* other than the fact that both organisms have a high G+C content in their genome. As expected, *E. coli*, a Gram-negative bacterium with a low G+C content, shares only 35% identity and 60% homology with the *S. griseus pyrB*, thus implying that these two organisms are less closely related.

Comparison of the *S. griseus* UPRTase sequence with other bacteria shows similar results. The *S. griseus* UPRTase sequence shares 70% identity and 95% homology with *M. tuberculosis* and 75% identity and 90% homology with *T. aquaticus*. Again, the high degree of AA sequence similarity seen with these organisms indicates that they may share a close phylogenetic relationship.

**Genetic organization.**

The pyrimidine genes were studied using polymerase chain reaction (PCR) in *S. griseus* 10137. Evidence available from PCR shows that *Streptomyces* may contain a gene organization of *pyrR* followed by *pyrB, pyrC, carA, carB*, and then *pyrF* in an operon similar to that found in
Mycobacterium tuberculosis. The PCR products obtained for S. griseus in this study, including the pyrR-pyrB products which have been confirmed by sequencing, have a size and orientation consistent with a gene organization of pyrRBC-carAB-pyrF. Comparison of these product sizes with those expected for M. tuberculosis also suggests that the open reading frame between pyrC and carA may be present in Streptomyces, and that an additional 1.2 kbp in length for the pyrR-pyrF, carA-pyrF, and carB-pyrF products suggests an additional open reading frame could be located between carB and pyrF. A likely candidate may be pyrD considering the presence of this gene in a similar location in the operon from Bacillus.

The operon arrangement is similar to that of operons described in other Gram-positive organisms including Bacillus, Lactobacillus, Enterococcus, and Mycobacterium (Fig. 2-3). Therefore, this grouping of pyrimidine biosynthetic genes in Streptomyces is not unexpected. In fact, using the same primers employed to deduce the pyrimidine operon in S. griseus, L. Hughes (1998) has found a pyrimidine gene operon organization in Streptomyces coelicolor that is identical to that described for S. griseus. It is common for bacteria to have all of the genes of a metabolic pathway organized in a cluster that is coordinately regulated. While further study is necessary to determine the organization of the entire operon (i.e., is
pyrD located within the operon and is pyrE located downstream?), this initial evidence is consistent with what would be expected given some of the other known evolutionary relationships of the genus.
REFERENCES


